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Of the genetic alterations associated with breast cancer, changes in p53 are the most frequent and identified. The overall goal of our studies is to identify small molecule compounds that bind and stabilize the subset of tumor-derived p53 mutants. We anticipate that the identification of such compounds will serve as a scaffold for the preparation of small molecule drugs for the treatment of p53-mediated breast cancer. Towards our goal we have employed a Multiple Solvent Crystal Structures (MSCS) technique to identify p53 binding sites for the small molecule compounds Tris and Isopropanol. The X-ray crystal structure of both complexes suggests that they each may repair a subset of tumor derived p53 mutants. Correlating with this possibility, we have shown that Tris stabilizes p53 structure in solution. In the coming year, we will focus on using the p53/isopropanol and p53/Tris complexes as scaffolds for developing high affinity compounds for the rescue of tumor-derived p53 mutations. We will specifically focus on using computational algorithms to screen virtual libraries for compounds that build upon the p53 interactions made by isopropanol and Tris compounds. Compounds that show favorable properties will be characterized both structurally and functionally.

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INTRODUCTION

Of the genetic alterations associated with breast cancer, changes in p53 are the most frequent and identified in 20-40% of all cases (Borresen-Dale, 2003; Ziyaie et al., 2000). In fact, approximately half of the major forms of cancer contain p53 mutations, and the vast majority of these cluster in conserved regions or "hot spots" (Hainaut and Hollstein, 2000). Missense mutations leading to amino acid changes are the most common p53 alterations in breast cancer, as in other tumors (Hainaut and Hollstein, 2000). Together, these observations suggest a requirement for a putative oncogenic contribution conferred by many TP53 mutations in breast cancer, and imply that the development of small molecule compounds that may bind and reactivate the protein product of tumor-derived TP53 mutations may have therapeutic use for the treatment of breast cancer.

The TP53 gene encodes the p53 protein that regulates the transcription of a number of genes involved in cell-cycle arrest and induction of apoptosis in response to cellular or genotoxic stress such as DNA damage or hypoxia (Bargonetti and Manfredi, 2002). The transcriptional activity of p53 is mediated by a tetrameric form of the protein that binds DNA in a sequence-specific fashion to activate or repress the transcription of target genes (El-Deiry et al., 1993; Friedman et al., 1993; Halazonetis and Kandil, 1993; Stenger et al., 1994). p53 contains four functionally distinct domains: a N-terminal transcriptional activation domain (residues 1 to 44), a central core (residues 102 to 292) containing a DNA binding domain, a tetramerization region (residues 320 to 356), and a regulatory domain (residues 356-393) (Cho et al., 1994; Pavletich et al., 1993; Wang et al., 1993). The vast majority of tumor-derived p53 mutations are localized to the p53 core domain (Cho et al., 1994). The X-ray crystal structure of the monomeric core domain of p53 bound to DNA has provided invaluable insights into how several tumor-derived mutations in p53 disrupt its activity (Cho et al., 1994). Specifically, these studies reveal that the tumor-derived p53 mutations that are localized to the core domain result in two different classes of p53 protein alterations: (1) reduced protein thermostability mutations and (2) mutations that directly disrupt protein-DNA contacts. Both classes of mutations functionally compromise the ability of p53 to carry out its normal tumor suppression function and thus contribute to neoplasia. The goal of our studies is to identify lead compounds that bind and stabilize the subset of tumor-derived stability mutants within the p53 core domain. We anticipate that the identification of such compounds will serve as a scaffold for the preparation of small molecule drugs for the treatment of p53-mediated breast cancer.

The Specific Aims of the proposal are to (1) Determine the high resolution X-ray crystal structure of the p53-core domain bound to a stabilizing peptide called FL-CDB3, (2) Use the Multiple Solvent Crystal Structures (MSCS) technique, to identify novel p53 stabilization sites, (3) Use the structural information of aims 1 and 2 as a scaffold for using computational strategies for the further development of small molecule compounds and peptides for the reactivation of tumor derived p53 mutants, and (4) Functionally characterize the p53-stabilizing and p53-

reactivation properties of the molecules derived from aim 3, and determine their structures in complex with p53.

BODY

During the first year of the funding period we completed Aim1 (Tasks 1-2), Aim2 (Tasks 3-4) and Task 7 of Aim 4. For Aim1, we determined the 2.5Å resolution structure of crystals that were prepared by mixing the p53 core domain with the Fl-CDB3 peptide. Unfortunately, the structure did not reveal ordered electron density for the peptide. Subsequent experiments involved soaking preformed p53 core domain crystals with peptide, which also produced a structure in which no ordered density for the peptide could be identified. We conclude that the FL-CDB3 peptide does not bind p53 in a unique location and conformation and therefore that it is not possible to characterize a structure of a p53/FL-CDB3 complex. This is consistent with recent observations that have been made by Fersht and coworkers (Friedler et al., 2005).

Figure 1. A) **Structure of the p53 core domain/Tris complex.** Left- Overall structure of the complex, Right – close-up of the complex highlighting omit-density (blue chicken wire) and protein-mediated hydrogen bonds (orange dotted lines) mediated by the Tris molecule. B) **Structure of the p53 core domain/isopropanol complex.** Left – Overall structure of the complex. Right – close-up of the complex using the same color-coding as in A. The red chicken wire represents displaced water molecules upon isoproponal binding.

For Aim 2, we have soaked crystals containing the p53 core domain with various small molecule compounds and have determined the structures of p53 soaked with these various compounds to identify possible small molecule binding sites. Using this strategy, we have identified p53 binding sites for the small molecule compounds isopropanol and Tris. The structures that we have determined are shown in Figure 1. Interestingly, both compounds interact with regions of the p53 core domain that have been implicated to mediate reduced stability and/or reduced DNA binding in a subset of tumor derived p53 mutants. Specifically, the structure of the p53 core domain bound to Tris shows that the molecule binds between the S1 and S10 strands of the core domain (Figure 1a). This Tris molecule makes numerous interactions with the protein that are reminiscent of stabilizing interactions made by the aspartic acid of a human N268D mutant that restored activity to several tumor-derived p53 mutant proteins (Nikolova, 2000). This correlation suggests that the Tris/p53 core domain structure may be useful as a scaffold for the design of related small molecule compounds that may increase the thermostability and activity of a subset of tumor-derived p53 mutants. The p53 core domain bound to isopropanol revels that the isopropanol binds to and fixes the orientation of the L1 loop, a region whose reduce flexibility has been correlated with increased p53 binding to DNA (Zhao et al., 2001) (Figure 1b). The isopropanol binding site may therefore also be exploited for the development of compounds that may stabilize the p53 core domain and/or introduce additional p53 core domain-DNA interactions that might compensate for the detrimental effect of a subset of tumor-derived p53 mutations.

In light of the observation that the small molecule compound Tris is bound to the p53 core domain in a region that is susceptible to tumor derived stability mutations and that mediates restored activity via the N268D mutation, we addressed whether Tris binding itself might increase p53 core domain stability (Task 7 of Aim4). In order to carry out experiments that would address this possibility, we carried out urea-induced denaturation studies of the p53 core domain in the absence and presence of 1 mM Tris. For these studies, we monitored the increase in fluorescence of a tryptophan residue within the p53 core domain as a function of urea-induced unfolding. Remarkably, we observe that while the unliganded p53 protein denatures at a urea concentration of 2.39 M (the inflection point between the fully folded and fully unfolded protein), the Tris-liganded p53 core domain denatures at a higher urea concentration of 2.61 M (Figure 2). A calculation of delta-delta G extrapolated to zero urea concentration reveals that Tris binding to the p53 core domain correlates with an increase in stability of 0.37

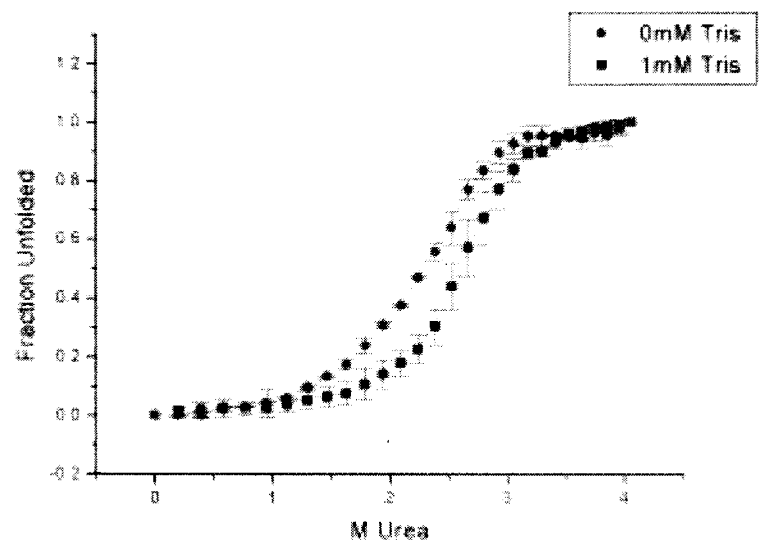

Figure 2. Urea induced unfolding of the p53 core domain in the absence (red) or presence of 1 mM Tris. Fraction unfolded is monitored by an increase of tryptophan florescence from a tryptophan that is buried in the folded protein.

kcal/mol. These results are highly encouraging and suggest that the small molecule compound Tris may be used as a scaffold to design p53 stabilization compounds.

KEY RESEARCH ACCOMPLISHMENTS

- We determined the high resolution crystal structures of the p53 core domain bound to the small molecule compounds isopropanol and Tris.
- We showed that Tris binding to the p53 core domain increases the stability of the core domain by 0.37 kcal/mole, suggesting that Tris is an attractive lead for the development of p53 stabilization compounds that may have therapeutic application for p53-mediate breast cancer.

REPORTABLE OUTCOMES

A manuscript describing these studies is in preparation.

CONCLUSIONS

In the coming year we will focus on using the p53/isopropanol and p53/Tris complexes as scaffolds for developing high affinity compounds for the rescue of tumor-derived p53 mutations. We will specifically focus on using computational algorithms to screen virtual libraries for compounds that build upon the p53 interactions made by isopropanol and Tris compounds (Aim 3). Compounds that appear promising will be obtained and assayed experimentally for their ability to bind to p53 and restore function to tumor-derived p53 mutant proteins (Task 7 of Aim 4). Compounds that show favorable properties will be structurally characterized for further analysis (Aim 4).

The structure-based drug design approach (often called "rational drug design"), which we are using towards the development of small molecule compounds that might restore function to tumor-derived p53 mutants, is a recently exploited and particularly powerful strategy which uses protein structural information to specifically design small peptides or non-peptidic molecules that modulate the activity of a protein of interest (Garrett and Workman, 1999; Huang, 2000; Jackson, 1997; Oakley and Wilce, 2000; Tada et al., 1999; Wang et al., 1999; Wieczorek et al., 1996). This strategy has shown considerable promise, already yielding clinically useful peptides and compounds (Amzel, 1998; Gane and Dean, 2000; Kirkpatrick et al., 1999; Klebe, 1998; Kubinyi, 1998; Lunney, 1998; Roe et al., 1998; Sehgal, 2002) as well as several other compounds currently in clinical trials (Klebe, 1998). <u>Based on our encouraging results to date, we propose that a structure-based approach is an effective strategy of achieving our ultimate goal of developing p53-targeting drugs that will have clinical application for the treatment of p53-mediated breast cancer.</u>

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